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Differential sensitivities of pulmonary and coronary arteries to hemoglobin-based oxygen carriers and nitrovasodilators: Study in a bovine ex vivo model of vascular strips

Vera Fonseca [a], Jessica Avizinis [a], Paula Moon-Massat [b,c], Daniel Freilich [b,c,d,e,f], Hae Won Kim [a], Chi-Ming Hai [a,*]

[a] Department of Molecular Pharmacology, Physiology & Biotechnology, Brown University, Providence, Rhode Island, USA
[b] Neurotrauma Department, Naval Medical Research Center, Silver Spring, Maryland, USA
[c] Henry Jackson Foundation, Rockville, Maryland, USA
[d] Infectious Diseases, National Naval Medical Center, Silver Spring, Maryland, USA
[e] Medicine Department, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA
[f] Surgery Department, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA

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ABSTRACT

Vasoconstriction is a major adverse effect of first and second generation hemoglobin-based oxygen carriers (HBOCs) that hinders their development as blood substitute. However, intravenous infusion of HBOC-201 (second generation) to patients induces significant pulmonary hypertension without significant coronary vasoconstriction. We compared contractile responses of isolated bovine pulmonary and coronary arterial strips to HBOC-201 and HBOC-205LLLT.MW600 (third generation), polymerized bovine hemoglobins of different molecular weight, and their attenuation by nitroglycerin, sodium nitroprusside (SNP), and sodium nitrite. Pulmonary arteries developed negligible basal tone, but exhibited HBOC-dependent amplification of phenylephrine-induced contractions. In contrast, coronary arteries developed significant basal tone, and exhibited HBOC-dependent constant force increment to serotonin-induced contractions. Therefore, relative to basal tone, HBOC-induced contractions were greater in pulmonary than coronary arteries. Furthermore, HBOC-205LLLT.MW600 appeared to be less vasoactive than HBOC-201. Unexpectedly, pulmonary and coronary arteries exhibited differential sensitivities to nitrovasodilators in parallel with their differential sensitivities to HBOC. However, SNP and sodium nitrite induced significant methemoglobin formation from HBOC, whereas nitroglycerin did not. These results suggest that phenotypic differences between pulmonary and coronary vascular smooth muscle cells could explain the differential hypertensive effects of HBOC on pulmonary and coronary circulation in patients. Among the three nitrovasodilators investigated, nitroglycerin appears to be the most promising candidate for attenuating HBOC-induced pulmonary hypertension in older HBOCs.

1. Introduction

Second generation HBOCs such as HBOC-201 have been found to be useful in certain clinical settings. For example, results from a multicenter phase III trial in elective orthopedic surgery indicated that HBOC-201 eliminated transfusion in the majority of subjects, suggesting that treatment with HBOC-201 is appropriate when any form of red blood cell is not an option (Jahr et al., 2008). In a recently published animal study, Rempf et al. (2009) showed that administration of bovine polymerized hemoglobin (HBOC-200) before and during coronary occlusion reduced infarct size in rabbits. As commented by Jahr (2009), the findings by Rempf et al. (2009) suggest that second generation HBOCs may be useful in certain clinical settings such as reduction of myocardial infarction size during myocardial ischemia. However, adverse effects of hemoglobin-based oxygen carriers (HBOCs) remain a major barrier to FDA approval for clinical application (Alayash, 2004; Estep et al., 2008; Natanson et al., 2008).

Vasoconstriction — a major manifestation of HBOC's adverse effects — is caused primarily by the scavenging of endothelium-derived nitric oxide (NO) by HBOC (Kim-Shapiro et al., 2006; Olson et al., 2004). However, a recently published clinical trial indicated that intravenous infusion of HBOC-201 to patients led to systemic and pulmonary hypertension without significant coronary vasoconstriction (Serruys et al., 2008). The authors suggested that autoregulatory mechanism of coronary circulation was not adversely affected by the infusion of HBOC-201. An alternative hypothesis is that the observed differential HBOC-sensitivity of pulmonary and coronary circulations reflects differential HBOC-sensitivity of pulmonary and coronary blood vessels, possibly due

* Corresponding author. Brown University, Box G-B3, Providence, RI 02912, USA. Tel.: +1 401 863 3288; fax: +1 401 863 1440.
E-mail address: Chi-Ming_Hai@brown.edu (C.-M. Hai).

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14. ABSTRACT

Vasoconstriction is a major adverse effect of first and second gener.ltion hemoglobin-based oxygen carriers (HBOCs) that hinders their deve lopment .1s blood substitute. However. intravenous infusion of HBOC-20 I (second generation) to patients induces significa nt pulmonary hypertension without significant coronary vasoconstriction. We compared contractile responses of isolated bovine pulmonary and coronary arterial strips to HBOC-201 and HBOC-205LLLT.MW600 (third generation). polymerized bovine hemoglobins of different molecular weight. and their attenuation by nitroglycerin, sodium nitroprusside (SNP). and sodium nitrite. Pu lmonary arteries developed negligible basal tone. but exhibited HBOC-dependent amplification of phenylephrine-induced contractions. In contrast, coronary arteries developed significant basal tone. and exhibited HBOC-dependent constant force increment to serotonin-induced cont ractions. Therefore, relative to bas,Jitone. HBOC-induced contr.1ctions were greater in pulmon,Jry tl1<1n coron,lry ,uteries. Furthermore. HBOC- 205LLLT.MW600 appeared to be less vasoactive than HBOC-201. Unexpectedly, pulmonary and coronary arteries exhibited differential sensitivities to nitrovasodil~tors in parallel with their differential sensitivities to HBOC. However, SNP and sodium nitrite induced significant methemoglobin formation from HIJOC. whereas nitroglycerin did not. These results suggest that phenorypic differences between pulmonary and coronary vascular smooth muscle cells could explain the differential hypertensive effects of HBOC on pulmonary and coronary circulation in patients. Among the three nitrovasodilators investigated. nitroglycerin appears to be the most promising candidate for ,menuating HBOC-induced pulmondry hypertension in older HBOCs.

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to two mechanisms: a) vessel type-specific different NO-sensitivity of vascular smooth muscle cells and/or b) vessel type-specific different levels of endothelium-derived NO. This hypothesis would predict that isolated pulmonary and coronary vessels should exhibit differential sensitivities to HBOC, and possibly to exogenous NO as released by nitrovasodilators. In this study, we tested this hypothesis by comparing contractile responses of bovine pulmonary and coronary arteries to HBOC-201 and HBOC-205LLLT.MW600 ex vivo, and their attenuation by nitroglycerin, sodium nitroprusside (SNP), and sodium nitrite. In addition, we sought to identify the best candidate among the three nitrovasodilators for attenuating HBOC-mediated contractions of pulmonary and coronary arteries.

Bovine pulmonary artery is a good model system for studying NO-scavenging by HBOC ex vivo, as this preparation has been used extensively in multiple studies that led to the discovery of NO as endothelium-derived relaxing factor (Ignarro et al., 1984, 1987, 1988a,b). HBOC-201 and HBOC-205LLLT.MW600 consist of different mixtures of native and polymerized bovine hemoglobins (Biopure Corporation; Dube et al., 2008). Major differences between these two HBOCs include average molecular weight (250 kD for HBOC-201 versus 600 kD for HBOC-205LLLT.MW600) and percentage of tetramers and dimers (0.8% and 1.4% in HBOC-201 versus 0.1% and 0.2% in HBOC-205LLLT.MW600). Average molecular weight is the major difference between these two preparations; thus, HBOC-205LT.LT.MW600 should be less likely to extravasate into the interstitial space and scavenge interstitial NO. HBOC-201 is currently approved for human use in South Africa and a related HBOC (Oxyglobin®, Hemoglobin glutamer 200 (bovine)) has been FDA and European Union approved in a veterinary application for canine anemia.

2. Material and methods

2.1. Tissue preparation

Bovine lungs, hearts, and portal vein connected to a small piece of liver, were collected from a slaughterhouse, and transported to the laboratory in ice-cold physiological salt solution (PSS) of the following composition (in mM): 140.1 NaCl, 4.7 KCl, 1.2 Na_2HPO_4, 2.0 MOPS (pH 7.4), 0.02 Na_2EDTA, 1.2 $MgSO_4$, 1.6 $CaCl_2$, and 5.6 D-glucose, as described previously (Kanefsky et al., 2006). Bovine organs were harvested in the morning of the day of an experiment, and used for experimentation within the day of the harvest. The procedure for preparing bovine pulmonary arterial strips was similar to that described by Ignarro et al. (1984). Briefly, second or third generation pulmonary arteries were carefully dissected from bovine lung. For the preparation of bovine coronary arterial strips, left anterior descending coronary arteries were excised from a bovine heart by carefully dissecting the surrounding adipose tissue away from cardiac muscle. Gross connective and adipose tissue were dissected away from isolated blood vessels using micro-dissecting scissors. Vascular strips (~4 mm in width) were then prepared from the blood vessels using a scalpel. Tubes of blood vessels are cut into rings, which were then cut open and hung such that the long axis of the vascular strip between the muscle clips was perpendicular to the long axis of the vascular tube. Vascular strips were used in this study in order to allow contact of the endothelial surface with HBOC.

2.2. Isometric contractions

The procedure for recording isometric contractions of arterial strips has been described previously (Kim et al., 2004). Briefly, one end of an arterial strip was clamped to a stainless steel clip connected to a force transducer (Grass FT.03; Quincy, MA), and the other end of the arterial strip was clamped to a stainless steel clip connected to a length manipulator (Narishige; Tokyo, Japan). Arterial strips were equilibrated for 2 h in PSS bubbled with air (37 °C, pH 7.4), and adjusted to reference length as described previously (An and Hai, 1999). Recent studies suggest that length–force relationship in smooth muscle is dynamic, such that the notion that smooth muscle possess a unique optimal length may be incorrect (Bai et al., 2004). Therefore, we set the length of a muscle strip by passive force, that is, the force exerted by a muscle strip immediately after a quick release. The protocol for setting the reference length consisted of first stretching a muscle strip so that total force was ~10 g and then releasing the muscle strip rapidly to a passive force that was ~2.5 g. Active force is total force minus passive force. After equilibration, each arterial strip was first stimulated for 10 min by K^+ depolarization with K-PSS, a solution similar in composition to PSS except that 104.95 mM NaCl was substituted for by an equimolar concentration of KCl. The active force (Fo) developed in this contraction was used as an internal control to normalize force developed by the same arterial strip in subsequent contractions. Arterial strips were then allowed to relax in PSS for 1 h before further experimentation.

2.3. Measurement of oxyhemoglobin, deoxyhemoglobin and methemoglobin

Solutions containing HBOC-201 and a nitrovasodilator (nitroglycerin, SNP, or sodium nitrite) were scanned for absorbance between 450 and 700 nm, in 1 nm intervals, using a spectrophotometer. The absorbance spectra were then analyzed by spectral deconvolution to calculate the concentrations of oxyhemoglobin, deoxyhemoglobin, and methemoglobin using a computer program, generously provided by Dr. Rakesh P. Patel, as described in Huang et al. (2005) and Isbell et al. (2007). The deconvolution program is a computer-based iteration routine designed to resolve the absorbance spectrum of a given hemoglobin solution into the different components of hemoglobin species based on the distinct absorbance spectra of individual hemoglobin species. Absorbance spectra of hemoglobin standard solutions and HBOC solutions are shown in the "Results" section.

2.4. Reagents

HBOC-201 (lot number RSH03C16), HBOC-205LLLT.MW600 (lot number ST0186-110907), and HBOC-201.LT.DL (lot number ST0186-100206) were provided by Biopure Corporation (Cambridge, MA). The HBOC-201 lot had the following specifications: hemoglobin 13.0 g/dL; methemoglobin 2%; oxyhemoglobin 2%; P_{50} 38 mm Hg; dimer (32 kD) 1.4%; tetramer (65 kD) 0.8%; high MW (>500 kD) 10.9%. The HBOC-205LLLT.MW600 lot had the following specification: hemoglobin 13.0 g/dL; methemoglobin 4%; oxyhemoglobin 3%; P_{50} 40 mm Hg; dimer (32 kD) 0.2%; tetramer (65 kD) 0.1%; high MW (>500 kD) 61%. The HBOC-201.LT.DL lot (purified HBOC-201) had the following specification: hemoglobin 13.2 g/dL; methemoglobin 2.6%; oxyhemoglobin 3.4%; P_{50} 42 mm Hg; dimer (32 kD) 0.22%; tetramer (65 kD) 0.17%; high MW (>500 kD) 11.4%. The formation of methemoglobin from HBOC in contact with air is a time-dependent process. Khan et al. (2003) observed insignificant increase in methemoglobin in an opened bag HBOC over the course of one day of research. However, Moallempour et al. (2009) and Osgood et al. (2005) have reported that prolonged exposure of HBOC to air for months would result in the formation of methemoglobin up to 65–80%. Therefore, we took the following precautions in the handling of HBOC: a) withdrawing HBOC from the storage bag without injecting air into the bag, b) limiting the maximum duration for using a given bag to 1 week, and c) storing HBOC at 4 °C. Phenylephrine HCl, L-arginine, serotonin, sodium nitrite, and SNP were purchased from Sigma. Nitroglycerin (5 mg/ml; lot number 7803) was purchased from American Regent (Shirley, NY).

2.5. Statistical analysis

Data are presented as means ± SE; n represents the number of arterial strips. IC_{50}s for nitrovasodilator-induced relaxations were

estimated by interpolation of concentration–response relations. We performed one-way analysis of variance (ANOVA) and calculation of the least significant difference for the comparison between each HBOC-treatment and untreated control ($p<0.05$ considered significant), as shown in Fig. 1A and Table 1. When necessary, we further confirmed the results from one-way ANOVA by two-way ANOVA with Tukey's test, as shown in Fig. 1B.

3. Results

3.1. HBOC-mediated contractile effects on bovine pulmonary and coronary arteries

Multiple studies have suggested that scavenging endogenous NO is the major mechanism of hemoglobin-induced vasoconstriction. For example, Hart et al. (1997) showed that L-nitro arginine eliminated the contractile responses of pre-contracted dog and rat vessels to diaspirin cross-linked hemoglobin, suggesting that the mechanism of diaspirin cross-linked hemoglobin-induced contractions was interference with NO. Ignarro et al. (1988a) have shown that, in endothelium-intact intrapulmonary arteries, oxyhemoglobin inhibited acetylcholine-induced relaxation and cGMP accumulation by 88%. In endothelium-denuded intrapulmonary arteries, Ignarro et al. (1988a) showed that oxyhemoglobin (1 μM) inhibited NO-induced relaxation by 91% and cGMP accumulation by 88%. In contrast, indomethacin failed to alter cGMP levels in intrapulmonary arteries the absence or presence of acetylcholine or NO (Ignarro et al., 1988a). Similarly, Vuylsteke et al. (2001) reported that indomethacin and BQ-123 (endothelin A receptor antagonist) had no effect on diaspirin cross-linked hemoglobin-induced contractions of rat mesenteric artery. Nevertheless, the possibility that other minor mechanisms may also contribute to HBOC-induced vasoconstriction cannot be completely excluded. In preliminary experiments, we removed the endothelium by scraping the luminal surface of an arterial segment with scalpel blade, and found that removal of the endothelium abolished HBOC-mediated contractile response, without affecting K^+-depolarization-induced contraction in bovine pulmonary arterial strips, indicating that an intact endothelium was necessary for observing HBOC-induced contractions (data not shown). Therefore, all experiments in this study were performed on intact arterial strips.

As shown in Fig. 1A (open circles), bovine pulmonary arterial strips contracted in response to phenylephrine in a concentration-dependent manner. At concentrations of phenylephrine below 1 μM, HBOC-201 (2 μM) had insignificant effect on phenylephrine-stimulated bovine pulmonary arteries (Fig. 1A, closed squares). However, at higher concentrations (1, 10, and 100 μM) of phenylephrine, HBOC-201 significantly enhanced contractions of phenylephrine-stimulated bovine pulmonary arteries. For example, at 1 μM phenylephrine, HBOC-201-treated pulmonary arterial strips developed active forces

Table 1
Nitrovasodilator-induced relaxation of untreated and HBOC-treated bovine pulmonary and coronary arteries.

Arteries	Treatment	IC_{50} (Log) Mean ± SE	IC_{50} (nM) (Mean)
Nitroglycerin			
Pulmonary Artery	PE	−8.66 ± 0.11 ($n=8$)	2.2
	PE + HBOC-201	−7.75 ± 0.09 ($n=10$)*	17.8
	PE + HBOC-205LL.LT.MW600	−7.98 ± 0.12 ($n=10$)*	10.5
Coronary Artery	Serotonin	−7.70 ± 0.19 ($n=9$)	20.0
	Serotonin + HBOC-201	−6.94 ± 0.12 ($n=10$)*	114.8
	Serotonin + HBOC-205LL.LT.MW600	−7.32 ± 0.16 ($n=10$)	47.9
Sodium nitroprusside			
Pulmonary Artery	PE	−8.94 ± 0.19 ($n=10$)	1.2
	PE + HBOC-201	−7.27 ± 0.14 ($n=10$)*	53.7
	PE + HBOC-205LL.LT.MW600	−7.94 ± 0.14 ($n=12$)*	11.5
Coronary Artery	Serotonin	−7.78 ± 0.12 ($n=8$)	16.6
	Serotonin + HBOC-201	−6.60 ± 0.04 ($n=8$)*	251.2
	Serotonin + HBOC-205LL.LT.MW600	−7.21 ± 0.09 ($n=8$)*	61.7
Sodium nitrite			
Pulmonary Artery	PE	−5.07 ± 0.34 ($n=6$)	8.5
	PE + HBOC-201	−3.98 ± 0.16 ($n=5$)*	104.7
	PE + HBOC-205LL.LT.MW600	−3.96 ± 0.21 ($n=6$)	109.6
Coronary Artery	Serotonin	−4.41 ± 0.08 ($n=12$)	38.9
	Serotonin + HBOC-201	−3.87 ± 0.09 ($n=12$)*	134.9
	Serotonin + HBOC-205LL.LT.MW600	−4.31 ± 0.10 ($n=12$)	49.0

Abbreviations: IC_{50} – concentration of nitrovasodilator that inhibits contractile force by 50%; n – number of animals; * – significantly different from untreated control ($p<0.05$), as determined by one-way ANOVA with the calculation of least significant difference. Concentrations of HBOC-201 and HBOC-205LL.LT.MW600 were both 2 μM; concentrations of phenylephrine (PE) and serotonin were both 10 μM.

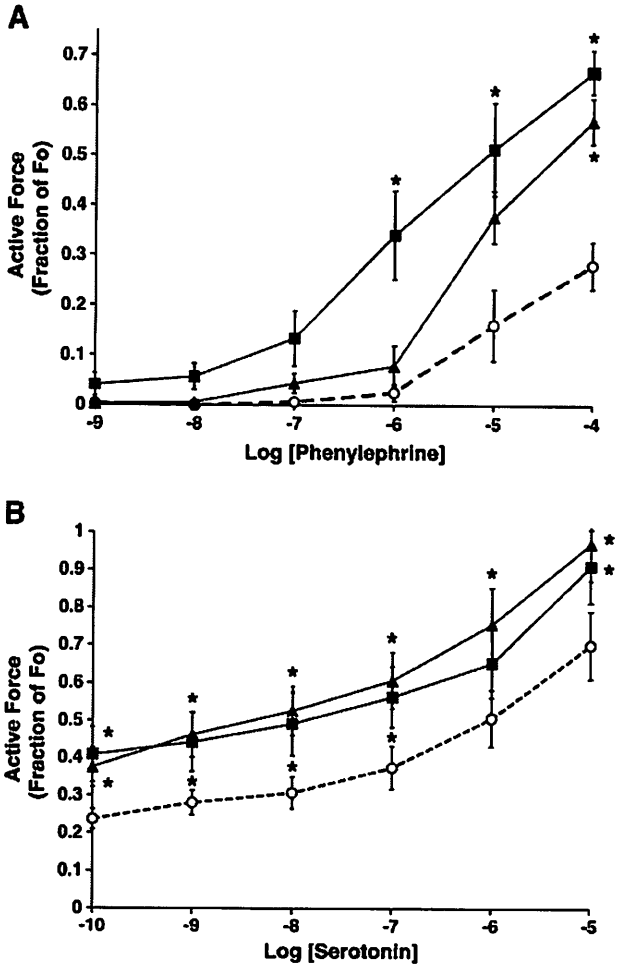

Fig. 1. HBOC-201 and HBOC-205LL.LT.MW600-mediated contractions in: A) bovine pulmonary arterial strips stimulated with phenylephrine and B) bovine coronary arterial strips stimulated with serotonin. Open circles, closed squares, and closed triangles represent phenylephrine or serotonin-stimulated contractions in the absence of HBOC, presence of 2 μM HBOC-201, and presence of 2 μM HBOC-205LL.LT.MW600, respectively. Active force is expressed as fraction of the maximum force (Fo) induced by 110 mM K^+-depolarization. Symbols represent mean, while vertical bars represent 1 SE ($n=4$ to 18). Asterisk (*) indicates significant difference between untreated and HBOC-treated groups ($p<0.05$).

that were approximately 14-fold of that developed by untreated pulmonary arterial strips. At 10 and 100 μM phenylephrine, the HBOC-201/untreated active force ratios were approximately 3.2 and 2.4-fold, respectively. Similarly, as shown in Fig. 1A (closed triangles), HBOC-205LLLT.MW600 (2 μM) had insignificant effect on phenylephrine-stimulated bovine pulmonary arteries at lower concentration of phenylephrine, but significantly enhanced contractions of phenylephrine-stimulated bovine pulmonary arteries at 100 μM phenylephrine. The HBOC-205LLLT.MW600/untreated active force ratio was approximately 2-fold at 100 μM phenylephrine.

Bovine coronary arteries were responsive to serotonin but non-responsive to phenylephrine. Therefore, serotonin was used for pre-stimulation of bovine coronary arteries in this study. We chose serotonin instead of other contractile agonists such as acetylcholine for this study, because serotonin is stored in platelets and released from platelets in contact with damaged endothelium, suggesting that serotonin is a pathophysiologically significant molecule in the coronary circulation (Jonnakuty and Gragnoli, 2008). As shown in Fig. 1B (open circles), serotonin stimulated the contraction of untreated bovine coronary arterial strips in a concentration-dependent manner. It is noteworthy that, even at very low concentration (0.1 nM) of serotonin, coronary arterial strips exhibited basal force, which was ~20% of maximum force that could be induced by K^+-depolarization. HBOC-201 (2 μM) and HBOC-205LLLT.MW600 (2 μM) both enhanced the contraction of serotonin-stimulated coronary arterial strips at all applied concentrations between 0.1 nM and 10 μM, as shown in Fig. 1B (closed symbols). One-way ANOVA did not indicate significant difference among the three groups in Fig. 1B. However, careful examination of Fig. 1B indicated that, at all tested serotonin concentrations, active forces developed by HBOC-treatment groups were consistently higher than those developed by untreated control. Therefore, we performed two-way ANOVA on the data shown in Fig. 1B with Tukey's test, which indicated significant difference between each HBOC-treatment group and untreated control at all tested serotonin concentrations, as shown in Fig. 1B. HBOC-induced force increment in coronary arterial strips was relatively constant, independent of serotonin concentration. At 0.1 nM serotonin, HBOC-201-treated coronary arterial strips developed active forces that were approximately 1.7-fold of that developed by untreated coronary arterial strips. At concentrations of serotonin ranging from 1 nM to 10 μM, the HBOC-201/untreated active force ratios ranged from 1.3 to 1.6-fold.

3.2. Nitroglycerin-induced attenuation of agonist and HBOC-stimulated contractions

In these experiments, vascular strips were first stimulated with 10 μM agonist (phenylephrine or serotonin) for 30 min, and then stimulated with 10 μM agonist in the presence of 2 μM HBOC-201 or 2 μM HBOC-205LLLT.MW600 for 1 h to allow contraction to reach steady state. Contracted vascular strips were then treated with nitroglycerin at concentrations ranging from 0.1 nM to 10 μM in the presence of the same concentrations of agonist and HBOC. For each concentration of nitroglycerin, a fresh solution was prepared, and 20 min were allowed for relaxation to reach steady state.

As shown in Fig. 2A (open circles), nitroglycerin induced relaxation of phenylephrine-stimulated, otherwise untreated, bovine pulmonary arterial strips in a concentration-dependent manner, with an average IC_{50} of 2.2 nM (Table 1). In comparison to untreated control, HBOC-201 and HBOC-205LLLT.MW600-treated arterial strips were less responsive to nitroglycerin (Fig. 2A, closed symbols), exhibiting significantly higher average IC_{50}s at 17.8 and 10.5 nM, respectively (Table 1).

As shown in Fig. 2B (open circles), nitroglycerin induced relaxation of serotonin-stimulated, otherwise untreated, bovine coronary arterial strips in a concentration-dependent manner, with an average IC_{50} of 20.0 nM (Table 1). In comparison to untreated control, HBOC-201

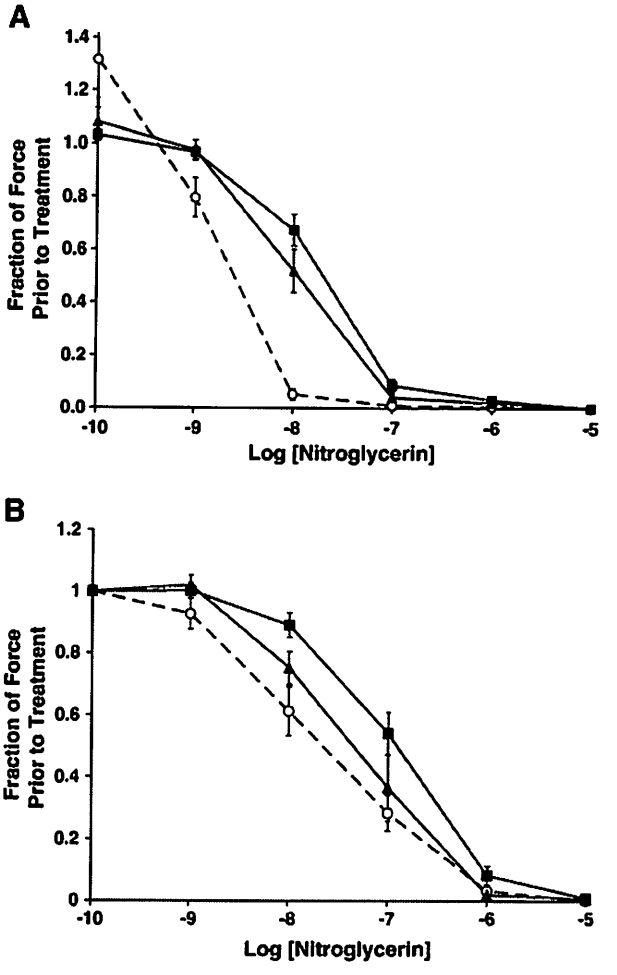

Fig. 2. Nitroglycerin-induced relaxation of untreated, HBOC-201-treated, and HBOC-205LLLT.MW600-treated: A) bovine pulmonary arterial strips stimulated with 10 μM phenylephrine and B) bovine coronary arterial strips stimulated with 10 μM serotonin. Open circles, closed squares, and closed triangles represent untreated control, 2 μM HBOC-201-treated tissues, and 2 μM HBOC-205LLLT.MW600-treated tissues, respectively. Active force after nitroglycerin treatment is expressed as fraction of the active force prior to nitroglycerin treatment. Symbols represent means, while vertical bars represents 1 SE ($n = 8$ to 14).

and HBOC-205LLLT.MW600-treated arterial strips appeared to be less responsive to nitroglycerin (Fig. 2B, closed symbols), exhibiting higher average IC_{50}s at 114.8 and 47.9 nM, respectively (Table 1). The average IC_{50} for the HBOC-201 group was significantly higher than control, but the IC_{50} for the HBOC-205LLLT.MW600 group was not significantly different from control.

3.3. SNP-induced attenuation of HBOC and agonist-stimulated contractions

As shown in Fig. 3A (open circles), SNP induced relaxation of phenylephrine-stimulated, otherwise untreated, bovine pulmonary arterial strips in a concentration-dependent manner, with an average IC_{50} of 1.2 nM (Table 1). In comparison to untreated control, HBOC-201 and HBOC-205LLLT.MW600-treated arterial strips were less responsive to SNP (Fig. 2A, closed symbols), exhibiting significantly higher average IC_{50}s at 53.7 and 11.5 nM, respectively (Table 1). The substantial difference in IC_{50} values among the control, HBOC-201, and HBOC-205LLLT.MW600 groups were also indicated by the relative shifting of the three concentration–response curves for SNP-mediated relaxations (Fig. 3A).

As shown in Fig. 3B (open circles), SNP induced relaxation of serotonin-stimulated, otherwise untreated, bovine coronary arterial

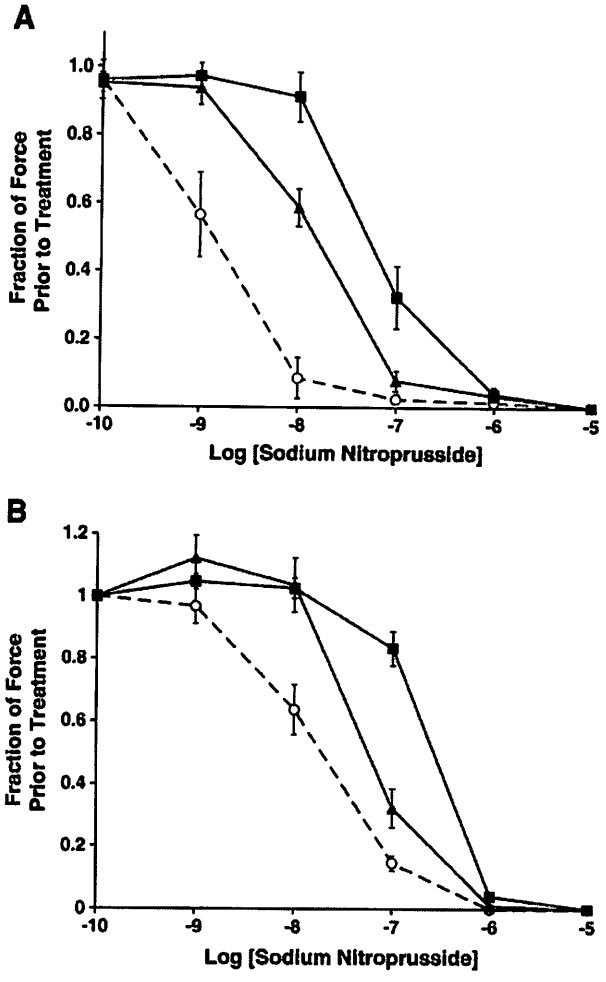

Fig. 3. SNP-induced relaxation of untreated, HBOC-201-treated, and HBOC-205LLLT.MW600-treated: A) bovine pulmonary arterial strips stimulated with 10 μM phenylephrine and B) bovine coronary arterial strips stimulated with 10 μM serotonin. Open circles, closed squares, and closed triangles represent untreated control, 2 μM HBOC-201-treated tissues, and 2 μM HBOC-205LLLT.MW600-treated tissues, respectively. Active force after SNP treatment is expressed as fraction of the active force prior to SNP treatment. Symbols represent means, while vertical bars represents 1 SE ($n = 8$ to 12).

strips in a concentration-dependent manner, with an average IC_{50} of 16.6 nM (Table 1). In comparison to untreated control, HBOC-201 and HBOC-205LLLT.MW600-treated arterial strips appeared to be less responsive to SNP (Fig. 3B, closed symbols), exhibiting significantly higher average IC_{50}s at 251.2 and 61.7 nM, respectively (Table 1).

3.4. Sodium nitrite-induced attenuation of HBOC and agonist-stimulated contractions

As shown in Fig. 4A (open circles), sodium nitrite induced relaxation of phenylephrine-stimulated, otherwise untreated, bovine pulmonary arterial strips in a concentration-dependent manner, with a relatively high average IC_{50} of 8.5 μM (Table 1). For bovine pulmonary artery, the IC_{50} for sodium nitrite was more than 1000-fold higher than the IC_{50}s for nitroglycerin and SNP (Table 1), indicating the relatively low potency of sodium nitrite as a vasodilator. In comparison to untreated control, HBOC-201 and HBOC-205LLLT.MW600-treated arterial strips were less responsive to sodium nitrite (Fig. 4A, closed symbols), exhibiting significantly and substantially higher average IC_{50} values at 104.7 and 109.6 μM, respectively (Table 1). The substantial difference in IC_{50} between the untreated and HBOC groups was also indicated by the relative shifting of the three concentration–response curves for sodium nitrite-mediated relaxations (Fig. 4A).

As shown in Fig. 4B (open circles), sodium nitrite induced relaxation of serotonin-stimulated, otherwise untreated, bovine coronary arterial strips in a concentration-dependent manner, with an average IC_{50} of 38.9 μM (Table 1). In comparison to untreated control, HBOC-201 and HBOC-205LLLT.MW600-treated arterial strips appeared to be less responsive to sodium nitrite (Fig. 4B, closed symbols), exhibiting higher average IC_{50}s at 134.9 and 49.0 μM, respectively (Table 1). The average IC_{50} for the HBOC-201 group was significantly higher than control, but the average IC_{50} for the HBOC-205LLLT.MW600 group was not significantly different from control.

3.5. Nitrovasodilator-induced methemoglobin formation from HBOC-201

The following experiments were performed to determine the effect of mixing HBOC-201 and nitrovasodilator on concentrations of total hemoglobin and methemoglobin, in parallel with the nitrovasodilator-mediated relaxation experiments. In these experiments, aliquots of solution containing HBOC-201 and a vasodilator (nitroglycerin, SNP, or sodium nitrite) were sampled from muscle chambers at time zero (immediately after) and 20 min after the preparation of the solution. Solutions were scanned for absorbance between 450 and 700 nm, in 1 nm intervals, using a spectrophotometer. The absorbance spectra were then analyzed by spectral deconvolution using a computer-based iteration routine, which resolves the absorbance spectrum of a given

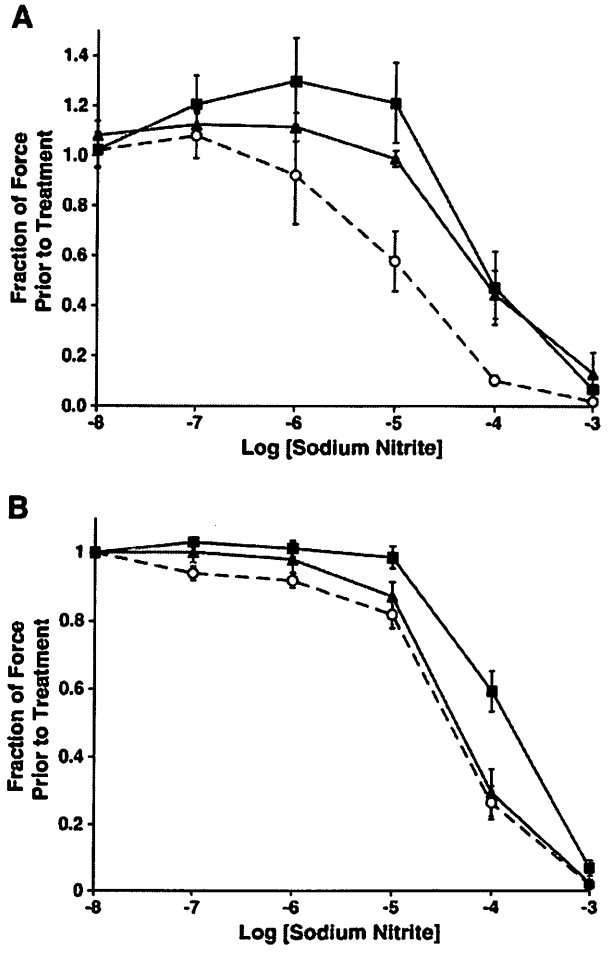

Fig. 4. Sodium nitrite-induced relaxing effects on untreated or HBOC-treated: A) bovine pulmonary arterial strips stimulated with 10 μM phenylephrine and B) bovine coronary arterial strips stimulated with 10 μM serotonin. Open circles, closed squares, and closed triangles represent untreated control, 2 μM HBOC-201-treated tissues, and 2 μM HBOC-205LLLT.MW600-treated tissues, respectively. Active force after sodium nitrite treatment is expressed as fraction of the active force prior to sodium nitrite treatment. Symbols represent means, while vertical bars represents 1 SE ($n = 5$ to 10).

hemoglobin solution into the different components of hemoglobin species based on the distinct absorbance spectra of individual hemoglobin species. For example, as shown in Fig. 5A, the absorbance of oxyhemoglobin is characterized by the two peaks of absorbance at 540 and 580 nm, whereas methemoglobin is characterized by multiple peaks of absorbance between 500 and 630 nm. Fig. 5B shows the absorbance spectra of two HBOC solutions having different concentrations of methemoglobin, deoxyhemoglobin, and oxyhemoglobin, but similar concentration of total hemoglobin (10 μM heme). As shown in Fig. 5B, changes in hemoglobin species are reflected in changes in absorbance spectrum consistent with the characteristic spectra of individual hemoglobin species. The concentrations of oxyhemoglobin, deoxyhemoglobin, and methemoglobin for the two absorbance spectra shown in Fig. 5B were resolved by the computer program.

Fig. 6 shows concentrations of total hemoglobin and methemoglobin as functions of nitrovasodilator concentration at time zero and 20 min after the change of a solution. In Fig. 6, the concentration ranges for the three nitrovasodilators were the same as those for studying the relaxing effects of these three nitrovasodilators, as shown previously in Figs. 2–4. As shown in Fig. 6A, total hemoglobin concentration remained relatively constant during the 20 min incubation period for all three nitrovasodilators. For example, at 10 μM nitroglycerin, total hemoglobin concentration at time zero (9.60 ± 0.44 μM) and 20 min (9.18 ± 0.18 μM) were not significantly different (Fig. 6A, open and closed circles). As shown in Fig. 6A, total hemoglobin concentration also did not change significantly with concentration of nitrovasodilator for all three nitrovasodilators. For example, in the nitroglycerin experiment, after 20 min of incubation, total hemoglobin concentration at 0.1 nM nitroglycerin (9.95 ± 0.21 μM) and 10 μM nitroglycerin (9.18 ± 0.18 μM) were not significantly different (Fig. 6A, closed circles).

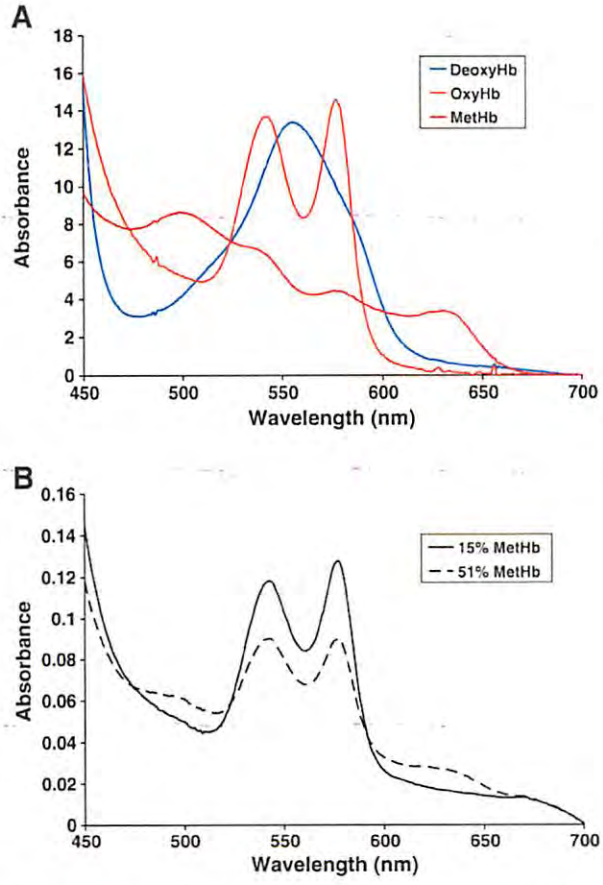

Fig. 5. Absorbance spectra of: A) standard solutions of deoxyhemoglobin (DeoxyHb; blue), oxyhemoglobin (OxyHb; red), and methemoglobin (MetHb; brown), and B) solutions of HBOC-201 containing similar total hemoglobin concentration (10 μM) but different concentrations of methemoglobin — 15% (solid line) and 51% (broken line). In panel B, the percentages of deoxyhemoglobin in the 15% and 51% methemoglobin solutions were 21 and 19%, respectively. The percentages of oxyhemoglobin in the 15% and 51% methemoglobin solutions were 64 and 30%, respectively. The absorbance spectra, as shown in panel A, are used by the spectral deconvolution program to resolve the concentrations of individual hemoglobin species in the two HBOC-201 solutions in panel B.

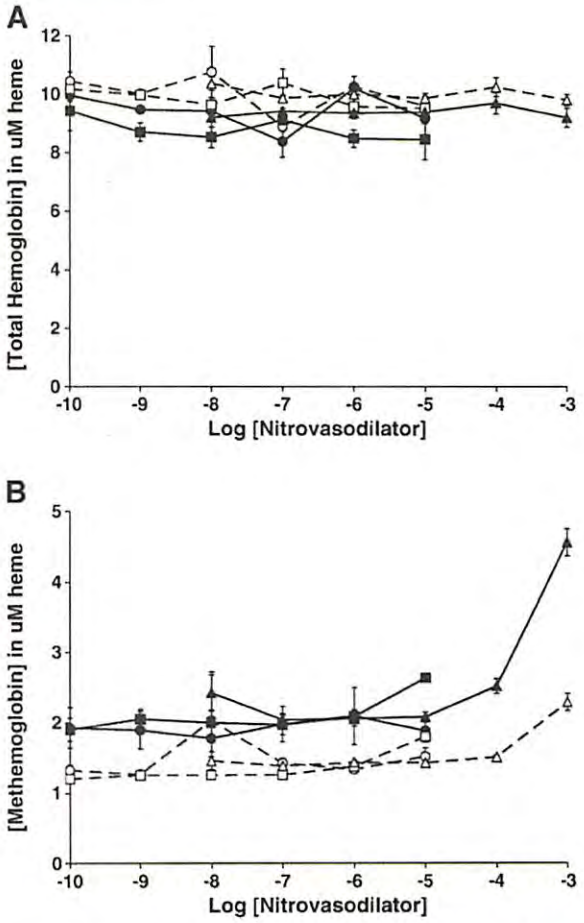

Fig. 6. Effects of mixing HBOC-201 with nitrovasodilator on: A) total hemoglobin concentration ([total hemoglobin]) and B) methemoglobin concentration ([methemoglobin]). In both panels, circles, squares, and triangles represent nitroglycerin data, SNP data, and sodium nitrite data, respectively. Open and closed symbols represent data collected at time zero (immediately after) and 20 min after the change of a solution, respectively. Concentrations of total hemoglobin and methemoglobin are expressed in μM heme. Symbols represent mean, while vertical bars represent 1 SE ($n = 3$).

As shown in Fig. 6B, methemoglobin formation was nitrovasodilator-specific and time-dependent during the 20 min incubation. For example, at 10 μM nitroglycerin, methemoglobin concentration at time zero (1.52 ± 0.12 μM) and 20 min (1.88 ± 0.16 μM) were not significantly different (Fig. 6B, open and closed circles). In contrast, at 10 μM SNP, methemoglobin concentration increased significantly from 1.80 ± 0.01 μM at time zero to 2.63 ± 0.04 μM at 20 min (Fig. 6B, open and closed squares). Similarly, at 1 mM sodium nitrite, methemoglobin concentration increased significantly from 2.29 ± 0.12 μM at time zero to 4.57 ± 0.19 μM at 20 min (Fig. 6B, open and closed triangles).

3.6. *Comparison between effects of HBOC-201 and HBOC-201.LT.DL (purified HBOC-201) in HBOC-induced contractions and their attenuation by SNP*

As shown previously in Figs. 1 and 3, HBOC-201 and HBOC205LLLT. MW600-treated pulmonary arterial strips appeared to exhibit different

concentration-dependencies of phenylephrine-induced contractions (Fig. 1A), and SNP-induced relaxations (Fig. 3A). Major differences between these two HBOC preparations include average molecular weight, and percentage of tetramers and dimers. To determine whether lowering percentages of tetramers and dimers in HBOC-201 alters the vasoactive property of HBOC-201, we compared the effects of HBOC-201 and HBOC-201.LT.DL in HBOC-induced contractions and their attenuation by SNP. HBOC-201.LT.DL is purified HBOC-201 that has relatively lower percentages of dimer (0.22%) and tetramer (0.17%). For comparing the vasoconstrictive effects of the two HBOCs, equilibrated pulmonary arterial strips were pre-stimulated with 10 μM phenylephrine for 30 min, and then stimulated with 2 μM HBOC-201 or 2 μM HBOC-201.LT.HBOC in the presence of 10 μM phenylephrine for 1 h to allow active force to reach steady state. As shown in Fig. 7A, the active forces developed by HBOC-201 and HBOC-201.LT.DL-treated arterial strips were not significantly different.

For comparing the antagonistic effects of the two HBOCs on nitrovasodilators, pulmonary arterial strips were pre-stimulated with 10 μM phenylephrine for 30 min, and then stimulated with 2 μM HBOC-201 or 2 μM HBOC-201.LT.DL in the presence of 10 μM phenylephrine for 1 h to allow active force to reach steady state. Contracted arterial strips were then treated with SNP at concentrations ranging from 0.1 nM to 10 μM in the presence of the same concentrations of phenylephrine and HBOC. A fresh solution was prepared for each concentration of SNP. Twenty minutes were allowed for the relaxation of arterial strips at each concentration of SNP. As shown in Fig. 7B, the concentration–response response relations of SNP-induced relaxations in HBOC-201 and HBOC-201.LT.DL-treated arterial strips were not significantly different.

3.7. Bovine portal vein

This vessel was included in this study to test the postulate that blood vessels that are insensitive to HBOC are also insensitive to nitrovasodilators. As shown in Fig. 8A (open circles). Neither HBOC-201 (2 μM) nor HBOC-205LLLT.MW600 (2 μM) significantly altered phenylephrine-stimulated contractions in bovine portal venous strips at all applied concentrations of phenylephrine between 0.1 nM and 10 μM. As shown in Fig. 8B, bovine portal venous strips were relatively insensitive to nitroglycerin. For example, the extent of relaxation of phenylephrine-stimulated, otherwise untreated induced by 10 μM nitroglycerin was only 39% (Fig. 8B, open circles). Similarly, the extent of relaxation induced by 10 μM nitroglycerin in HBOC-201 and HBOC-205LLLT.MW600-treated, phenylephrine-stimulated portal venous strips were only 46% and 41%, respectively (Fig. 8B, closed symbols). Similar results were obtained with SNP and sodium nitrite.

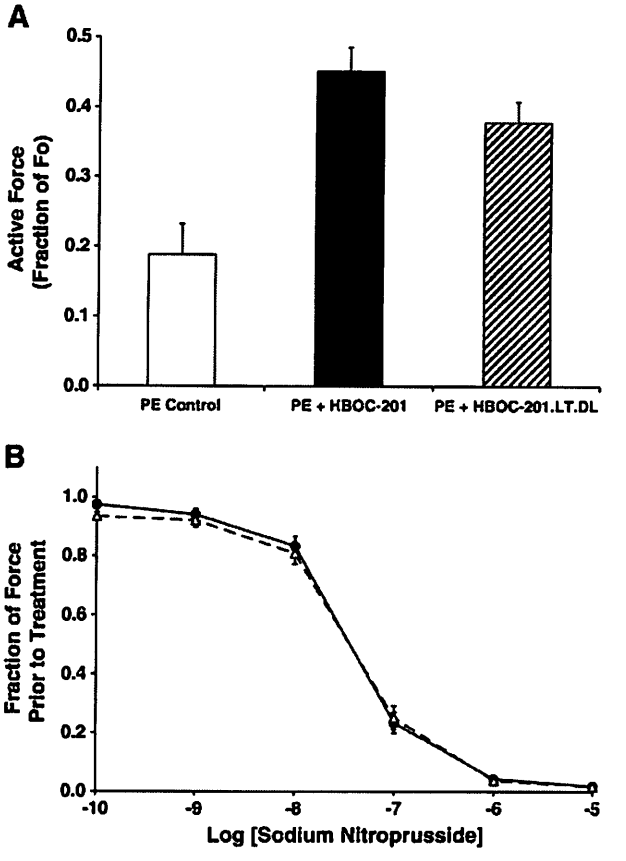

Fig. 7. Comparison between HBOC-201 and HBOC-201.LT.DL (purified HBOC-201) in: (A) HBOC-induced contractions, and (B) SNP-induced relaxations in bovine pulmonary arterial strips. In panel A, active force is expressed as fraction of the maximum force (Fo) induced by 110 mM K^+-depolarization. In panel B, closed circles represent 2 μM HBOC-201-treated, 10 μM phenylephrine-stimulated tissues; and open triangles represent 2 μM HBOC-205LLLT.MW600-treated, 10 μM phenylephrine-stimulated tissues. Active force after SNP treatment is expressed as fraction of the active force prior to SNP treatment. Symbols represent mean, while vertical bars represent 1 SE ($n = 11$ to 20).

4. Discussion

Vasoconstriction is a major adverse effect of first and second generation hemoglobin-based oxygen carriers (HBOCs) that hinders their acceptance as blood substitute(s). However, pulmonary and coronary circulations exhibit differential sensitivities to HBOC (Serruys et al., 2008). In this study, we tested the hypothesis that the observed differential HBOC-sensitivity of pulmonary and coronary circulations reflects differential HBOC-sensitivity of pulmonary and coronary blood vessels by comparing contractile responses of bovine pulmonary and coronary arteries to HBOC-201 and HBOC-205LLLT.MW600, and their attenuation by nitroglycerin, sodium nitroprusside (SNP), and sodium nitrite. We determined that bovine pulmonary and coronary arteries exhibited distinct characteristics of HBOC-mediated contractions (Fig. 1). Pulmonary arteries developed negligible basal tone, but exhibited HBOC-dependent potentiation of phenylephrine-induced contractions. In contrast, coronary arteries developed significant basal tone, and exhibited HBOC-dependent constant force increment to serotonin-induced contractions. Basal tone appears to be a characteristic of coronary circulation and mediated by multiple mechanisms, including stretch-activated cation channels, signaling protein kinases, and reactive oxygen species (Liu and Gutterman, 2009). As shown in Fig. 1, relative to basal tone, HBOC-induced contractions were greater in pulmonary than coronary arteries. For example, the HBOC-201-treated/untreated active force ratios for bovine pulmonary arteries were relatively high, ranging from 2.4 to 14-fold, whereas the HBOC-201/untreated active force ratios for bovine coronary arteries were relatively low, ranging from 1.3-fold to 1.7-fold (Fig. 1). These results suggest that phenotypic differences between pulmonary and coronary vascular smooth muscle could explain the differential hypertensive effects of HBOC on pulmonary and coronary circulations in patients (Serruys et al., 2008). However, while results from this study are consistent with the observed differential sensitivities of the systemic and pulmonary circulations to HBOC in vivo, the present study is not designed to examine the effects of HBOC on the microcirculation of these two vascular beds.

Unexpectedly, bovine pulmonary and coronary arteries exhibited different sensitivities to nitrovasodilators, in parallel with their differential sensitivities to HBOC (Figs. 2–4). For example, the IC_{50}s for nitroglycerin ranged from 2.2 to 18 nM for bovine pulmonary artery, but ranged from 20 to 115 nM for bovine coronary artery (Table 1). Similarly, the IC_{50}s for SNP ranged from 1.2 to 54 nM for bovine pulmonary artery, but ranged from 17 to 250 nM for bovine coronary artery (Table 1). Sodium nitrite was considerably less potent than

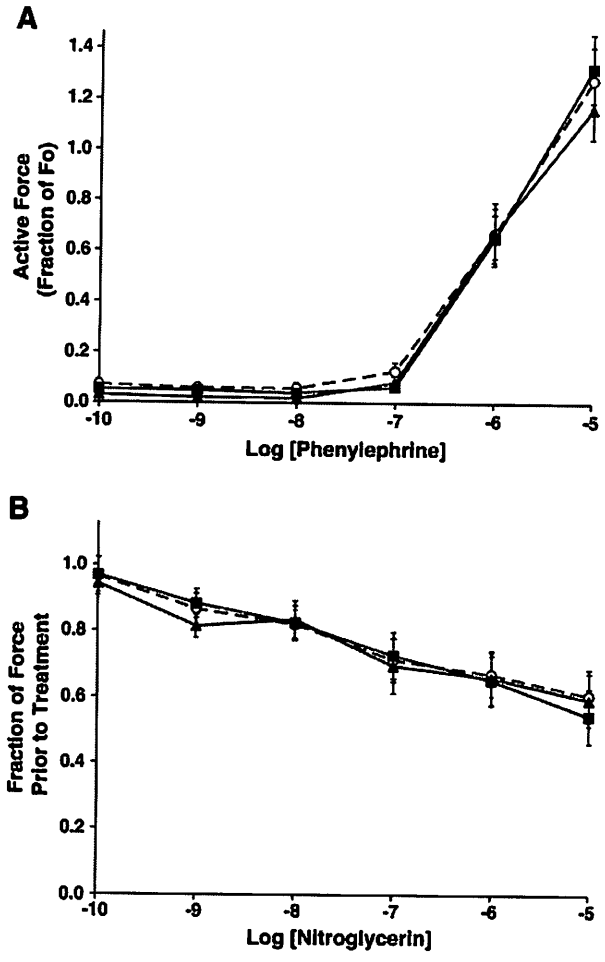

Fig. 8. Portal vein is insensitive to both HBOC and nitroglycerin. (A) HBOC-201 and HBOC-205LLLT.MW600-mediated contractions. Open circles, closed squares, and closed triangles represent phenylephrine-stimulated contractions in the absence of HBOC, presence of 2 μM HBOC-201, and the presence of 2 μM HBOC-205LLLT.MW600, respectively. Active force is expressed as fraction of the maximum force (Fo) induced by 110 mM K^+-depolarization. Symbols represent mean, while vertical bars represent 1 SE ($n = 18$). Asterisk (*) indicates significant difference between untreated and HBOC-treated groups ($p < 0.05$). (B) Nitroglycerin-induced relaxation of untreated, HBOC-201-treated, and HBOC-205LLLT.MW600-treated portal venous strips. Open circles, closed squares, and closed triangles represent untreated control, 2 μM HBOC-201-treated tissues, and 2 μM HBOC-205LLLT.MW600-treated tissues, respectively. Active force after nitroglycerin treatment is expressed as fraction of the active force prior to nitroglycerin treatment. Symbols represent means, while vertical bars represents 1 SE ($n = 13$ to 14).

nitroglycerin and SNP in relaxing HBOC and agonist-stimulated pulmonary and coronary arteries. Even at 1 mM, sodium nitrite induced only partial relaxation of agonist and HBOC-mediated contractions of bovine pulmonary and coronary arterial strips (Fig. 4). Altogether, these observations indicate that bovine pulmonary artery was more sensitive than bovine coronary artery in developing relaxation in response to nitrovasodilators. The expression of the NO-regulated enzyme guanylate cyclase is known to be vessel type-specific (Edwards et al., 1984; Schermuly et al., 2008). We postulate that blood vessels expressing higher levels of NO-regulated enzymes in vascular smooth muscle cells are more responsive to endothelium-derived NO, and therefore also more sensitive to NO-scavenging by HBOC, as well as more sensitive to exogenous NO as released by nitrovasodilators. Results from portal vein — a blood vessel that exhibits insensitivity to both HBOC and nitroglycerin — appear to support this postulate. This postulate suggests the intriguing possibility that nitrovasodilators may preferentially target HBOC-sensitive vascular beds.

As shown in Table 1, HBOC-treatment increased the IC_{50}s for nitrovasodilator-induced relaxations, presumably by scavenging NO and thereby reducing the availability of NO to vascular smooth muscle cells. However, IC_{50}s for the three nitrovasodilators — nitroglycerin, SNP, and sodium nitrite —exhibited different degrees of sensitivities to HBOC. For example, in the bovine pulmonary artery, the $IC_{50(HBOC\text{-}201)}/IC_{50(Control)}$ ratios for nitroglycerin, SNP, and sodium nitrite were 8.1, 44.8, and 12.3, respectively (Table 1), suggesting nitrovasodilator-specific differential availability of NO for scavenging by HBOC. Since nitroglycerin requires cellular bioactivation for releasing NO, nitroglycerin-derived NO may not be available in the extracellular fluid for scavenging by HBOC (Chen et al., 2005). In contrast, SNP in solution spontaneously releases NO, therefore, SNP-derived NO may be directly available for scavenging by HBOC in the extracellular fluid (Katsumi et al., 2007). The release of NO from nitrite is not completely understood, but nitrite has been shown to be a metabolic intermediate in the bioactivation of nitroglycerin to form NO (Lundberg and Weitzberg, 2005). Hemoglobin has been shown to function as nitrite reductase in converting sodium nitrite to NO under hypoxic conditions (Huang et al., 2005; Gladwin and Kim-Shapiro, 2008; Lundberg et al., 2008). In this study, HBOC-containing solutions were oxygenated with air; therefore, this mechanism probably plays a minor role in this study. The above analysis suggests that, among the three nitrovasodilators tested in this study, nitroglycerin-induced relaxation was least affected by HBOC-201.

HBOC-205LLLT.MW600 appeared to be less vasoactive than HBOC-201 in bovine pulmonary arteries (Figs. 1 and 3). However, we discovered that decreasing the dimer/tetramer content of HBOC-201 by purification did not significantly alter the HBOCs effect on contractions and their attenuation by SNP in bovine pulmonary arteries (Fig. 7). This finding is consistent with the report by Rice et al. (2008) that lowering tetramer content of HBOC-201 from 31% to 2% significantly decreased vasoactive responses, but further purification to 0.4% had no additional hemodynamic effect in severe swine hemorrhagic shock. Therefore, a higher molecular weight appears to be an important determinant of the lower vasoactivity of HBOC-205LLLT.MW600. However, paired experiments are needed to differentiate the vasoactivity of HBOC-201 and HBOC-205LLLT.MW600 in future studies.

Nitroglycerin, SNP, and sodium nitrite induced different extent of methemoglobin formation from HBOC-201. For nitroglycerin, within the efficacious concentration range for relaxation, methemoglobin formation from HBOC-201 was relatively independent of the concentration of nitroglycerin (Fig. 6B), suggesting that nitroglycerin-induced methemoglobin formation was insignificant compared to time-dependent HBOC autooxidation (Aranda et al., 2009; Shikama, 2006). In contrast, for SNP and sodium nitrite, within the efficacious concentration range for relaxation, methemoglobin formation was dependent on nitrovasodilator concentration, suggesting SNP and sodium nitrite-induced autocatalytic conversion of oxyhemoglobin to methemoglobin at normoxic PO_2 (Kim-Shapiro et al., 2005). Therefore, among the three vasodilators tested in this study, nitroglycerin is least reactive with HBOC-201 in methemoglobin formation.

In conclusion, results from this study suggest that phenotypic differences between pulmonary and coronary vascular smooth muscle cells may explain the differential hypertensive effects of HBOC on pulmonary and coronary circulation in patients. In consideration of the three vasodilators' differential potencies in attenuating HBOC-mediated contractions, and their differential reactivities with HBOC in methemoglobin formation, nitroglycerin appears to be the most promising candidate for attenuating HBOC-mediated hypertension.

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providing the HBOCs for this study. We thank Dr. Rakesh P. Patel for providing the computer program for analyzing the absorbance spectra of HBOC solutions by spectral deconvolution. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or the U.S. Government. CAPT. Freilich is a military service member (or employee of the U.S. Government). This work was prepared as part of his official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. §101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

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